Innervation of ectopic endometrium in a rat model of endometriosis

Karen J. Berkley*†, Natalia Dmitrieva*, Kathleen S. Curtis*, and Raymond E. Papka‡

*Program in Neuroscience, Florida State University, Tallahassee, FL 32306-1270; and ‡Department of Neurobiology, Northeastern Ohio Universities College of Medicine, Rootstown, OH 44272

Edited by Linda M. Bartoshuk, Yale University School of Medicine, New Haven, CT, and approved June 16, 2004 (received for review May 24, 2004)

Endometriosis (ENDO) is a disorder in which vascularized growths of endometrial tissue occur outside the uterus. Its symptoms include reduced fertility and severe pelvic pain. Mechanisms that maintain the ectopic growths and evoke symptoms are poorly understood. One factor not yet considered is that the ectopic growths develop their own innervation. Here, we tested the hypothesis that the growths develop both an autonomic and a sensory innervation. We used a rat model of surgically induced ENDO whose growths mimic those in women. Furthermore, similar to women with ENDO, such rats exhibit reduced fertility and increased pelvic nociception. The ENDO was induced by autotransplanting, on mesenteric cascade arteries, small pieces of uterus that formed vascularized cysts. The cysts and healthy uterus were harvested from proestrous rats and immunostained using the pan-neuronal marker PGP9.5 and specific markers for calcitonin gene-related peptide (CGRP) (sensory C and Aδ fibers), substance P (SP) (sensory C and Aδ fibers) and vesicular monoamine transporter (sympathetic fibers). Cysts (like the uterus) were robustly innervated, with many PGP9.5-stained neurites accompanying blood vessels and extending into nearby luminal epithelial layers. CGRP-, SP-, and vesicular monoamine transporter-immunostained neurites also were observed, with CGRP and SP neurites extending the furthest into the cyst lining. These results demonstrate that ectopic endometrial growths develop an autonomic and sensory innervation. This innervation could contribute not only to symptoms associated with ENDO but also to maintenance of the ectopic growths.

uterus | fertility | pain | transplant | neuropeptides

Endometriosis (ENDO) is a disorder in which viable growths of endometrial tissue occur outside the uterus, usually in the abdominal/pelvic cavity. Its symptoms include reduced fertility and severe pelvic pains such as dysmenorrhea, dyspareunia, dyschesia, and chronic pelvic pain (1). Considerable research interest centers on how ectopic endometrial growths in women implant and are maintained. Most researchers agree that the source of viable ectopic endometrial tissue is eutopic uterine tissue that escapes into the abdominal–pelvic cavity via retrograde menstruation (1). Current studies have therefore focused on molecular interactions between presumed strayed and possibly abnormal endometrial cells and their potential targets on organs or peritoneal tissue (1). Other studies concern mechanisms underlying the symptoms associated with ENDO. Regarding pain, most studies logically focus on ectopic growths. Although some investigations failed to find a correlation between pain scores and various aspects of the anatomy and biochemistry of the growths (1, 2), others found consistent correlations between pain and the depth of "infiltration" into peritoneum and pelvic organs or with substances that the growths or neighboring tissues release into peritoneal fluid (3, 4). Unfortunately, despite this research, a clear understanding of the mechanisms underlying development and maintenance of the ectopic growths and their associated symptoms remains elusive (1).

One potential contributor that has received virtually no attention is direct involvement of the nervous system. This omission is surprising, because ectopic growths could be conceptualized as vascularized autotransplants (4). It is well known that as organ or tissue transplants become vascularized, they become innervated, presumably via sprouting of para- and perivascular nerve fibers (5). Only two studies, both using human tissue, considered neural involvement in ENDO. One used a neurofilament marker and reported that the distance between nerve fibers and the ectopic endometrial growths tended to be less in women with pelvic pain than in those with no pain (6). Another group used S-100 protein as a marker for nerve fibers to compare samples of deeply infiltrating adenomyotic endometrial growths with other growths. The percentage of patients complaining of pain was greater in women with adenomyotic growths than other growths, and the adenomyotic growths were more likely to infiltrate nerves (7). No study, however, has investigated whether the ectopic growths develop their own nerve supply.

A rat model of surgically induced ENDO was developed in 1985 that involves autotransplantation of biopsies of uterus, or fat in controls, in the abdomen (ref. 8 and Fig. 1*A*). The uterine, but not fat, transplants become vascularized and form cysts that grow rapidly during the first month, stabilizing by 2 months and remaining viable for >10 months (8). Like women with ENDO, rats with cysts (but not the controls) display reduced fertility (8). They also display one of the symptoms of increased pelvic pain in women, that is, vaginal hyperalgesia (9). Furthermore, the ectopic cysts in rats bear clear similarities to human ectopic endometriotic growths. Specifically, the cysts in rats and the growths in women respond similarly to steroids, and the cysts synthesize or induce synthesis by cultured peritoneal cells of many of the same abnormal substances found in the ectopic endometrial growths and peritoneal fluid of women with ENDO (10). This rat model, therefore, seems suitable for investigating new ideas concerning mechanisms underlying signs and symptoms of ENDO (9, 10). Accordingly, here we tested the hypothesis that the cysts in this rat model develop their own nerve supply and then determined whether that supply included both sensory and sympathetic efferent fibers.

Materials and Methods

Animals. Adult female Sprague–Dawley rats (Charles River Breeding Laboratories) were used. They were housed individually, with ad libitum access to rat chow and water, and maintained under controlled conditions (24°C, 12:12 light/dark cycle with lights on at 7:00 a.m.). The estrous stage was monitored daily by vaginal lavage 2 h after lights on, beginning at least 2 weeks before surgery and continuing until the day of death. Only

This paper was submitted directly (Track II) to the PNAS office.

Freely available online through the PNAS open access option.

Abbreviations: CGRP, calcitonin gene-related peptide; ENDO, endometriosis; SP, substance P; VMAT, vesicular monoamine transporter.

†To whom correspondence should be addressed. E-mail: kberkley@psy.fsu.edu.

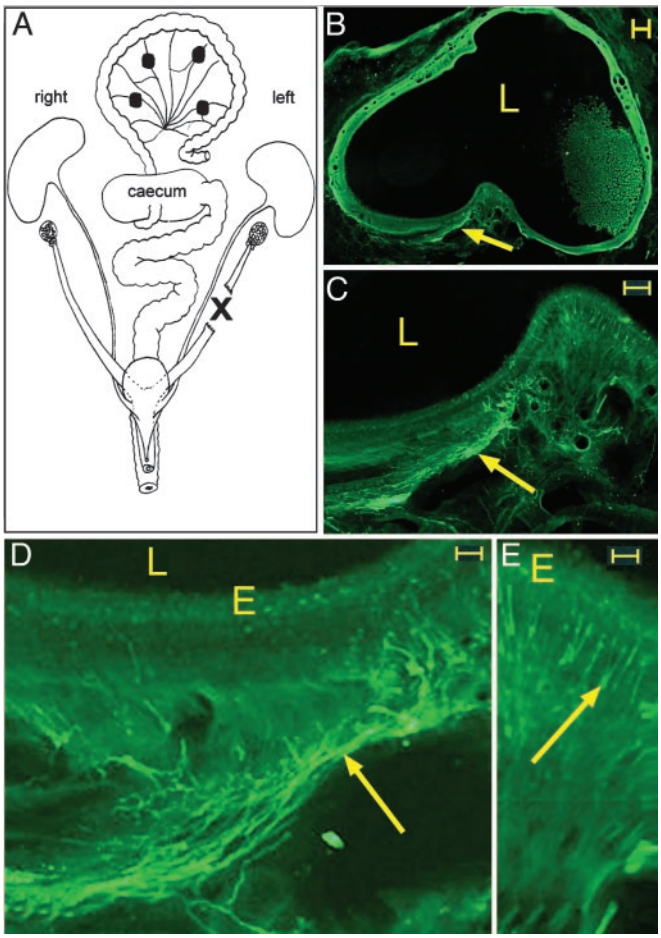

Fig. 1. Surgical model of ENDO (*A*) and sections through a cyst immunostained for the pan-neuronal marker PGP9.5 (*B–E*). (*A*) Diagram shows the surgical procedure: a partial hysterectomy is carried out (X), and 2 × 2-mm squares of uterine horn are sewn around alternate mesenteric cascade arteries (black dots). (*B*) Low-magnification digital image of a section through the cyst and lumen (L); note the bundle of nerves entering the hilus (arrow). (*C*) Higher-magnification view of the hilus area in *B*. Note the nerves entering the cyst wall (arrow). (*D*) Nerves (arrows) coursing through the cyst wall to the endometrium and approaching the epithelium (E) lining the cyst lumen (L). (*E*) Higher-magnification view of another part of the cyst wall in *B*. Note the individual neurites (arrows) in the endometrial layer and approaching the epithelium (E). (Bars: *B* and *C*, 250 μm; *D* and *E*, 25 μm.)

rats with regular 4-day cycles both before and after surgery were used. Rats weighed ≈225 g at the time of the ENDO surgery and ≈300 g when the cysts were harvested.

Surgical Procedures (ENDO). Surgery was done under aseptic precautions. Rats in estrus were anesthetized with a mixture of ketamine hydrochloride and xylazine (73 mg/kg and 8.8 mg/kg, respectively, i.p.). A midline abdominal incision exposed the uterus, and a 1-cm segment of the middle of the left uterine horn was removed and placed in warm sterile saline. Four pieces of uterine horn (≈2 × 2 mm) were cut from this segment and sewn, using 4.0 nylon sutures, around alternate cascade mesenteric arteries that supply the caudal small intestine, starting from the caecum (Fig. 1*A*). The incision was closed in layers, and the rat allowed to recover from anesthesia under close observation. Postoperative recovery was uneventful, and regular estrous cyclicity resumed within ≈1 week. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 86-23), revised in 1985. During all experiments, efforts were made to minimize both the animal's suffering and the number of rats used.

Tissue Harvesting and Immunohistochemistry. Rats were killed when cysts were known to be fully grown (≈7.5 weeks postsurgery; ref. 8), when they were in proestrus, the estrous stage in which ovarian hormonal levels are highest and the severity of vaginal hyperalgesia is greatest (9). They were anesthetized with urethane (1.2 g/kg, i.p.) and perfused transcardially first with saline then with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. The cysts and a 1-cm section of the middle of the healthy right uterine horn (i.e., the same area that had been taken from left uterine horn and used for the transplants) were harvested and stored in the fixative solution.

Samples were cryoprotected in 30% sucrose in 0.1 M phosphate buffer, frozen, serially sectioned on a cryostat (50 μm thick for PGP9.5, 16 μm thick for the other markers), and thaw mounted on glass slides. They were then immunostained using antibodies for the pan neuronal marker PGP9.5, vesicular monoamine transporter (VMAT) as a marker for sympathetic efferent fibers, and calcitonin gene-related peptide (CGRP) and substance P (SP) as markers for C and Aδ sensory fibers (11, 12) by standard techniques used routinely in our laboratories. Briefly, sections of cysts were incubated for 16–24 h at room temperature with primary antibodies: rabbit antibodies generated against PGP9.5 (dilution 1:6,000; Chemicon), CGRP (dilution 1:800; gift of Cary Cooper, University of Texas Medical Branch, Galveston), VMAT2 (dilution 1:800; Chemicon), and a guinea pig-generated antibody against SP (dilution 1:800; gift of Catia Sternini, Center for Ulcer Research and Education, University of California, Los Angeles). Sections were washed in PBS, incubated in a mixture of appropriate secondary antibodies labeled with Cy2-conjugated goat anti-rabbit IgG for PGP9.5 for 6 h (dilution 1:200; Jackson ImmunoResearch) or Alexa 488 for 1 h (all other markers, Molecular Probes) and mounted in PBS/glycerol. The SP and CGRP antibodies are well characterized (13–15) and have been used extensively in our previous studies. CGRP and SP are generally considered to be specific markers for C and Aδ sensory fibers (11, 12). In contrast, VMAT2 is expressed in monoamine-containing neurons and fibers, including sympathetic nerves (16), and the antibody produces immunostaining similar to that produced by other markers for sympathetic nerves (e.g., tyrosine hydroxylase; ref. 15). Controls included omission of the primary antiserum, omission of the secondary antibody, and absorption of the primary antiserum with its respective antigen (10 μg/ml diluted antiserum).

Images of sections immunostained for PGP9.5 were obtained digitally with an Optronics Microfire (Optronics International, Chelmsford, MA) camera and a MicroBrightField/Neurolucida system (MBF Laboratories, Williston, VT) with epifluorescence microscopy and fluorescein optics. Images of sections immunostained for SP, CGRP, and VMAT were obtained with an Olympus Fluoview Confocal laser-scanning microscope and with an Olympus Provis Microscope using epifluorescence microscopy. Images (digital and electronic) were imported into PHOTOSHOP V.6.0 (Adobe Systems, San Jose, CA), contrast and brightness adjusted if necessary, labeled, and printed.

Results

General. As observed by others (8–10), the ectopic growths were embedded in a large amount of fat and connective tissue. When dissected free, the growths grossly appeared as oval fluid-filled cysts, 3–10 mm in their largest diameter, each still attached to the blood vessel to which it had been sewn (Fig. 1*A*). When removed and sectioned, the cysts exhibited a lumen filled with leukocytes, lymphocytes, and plasma cells and

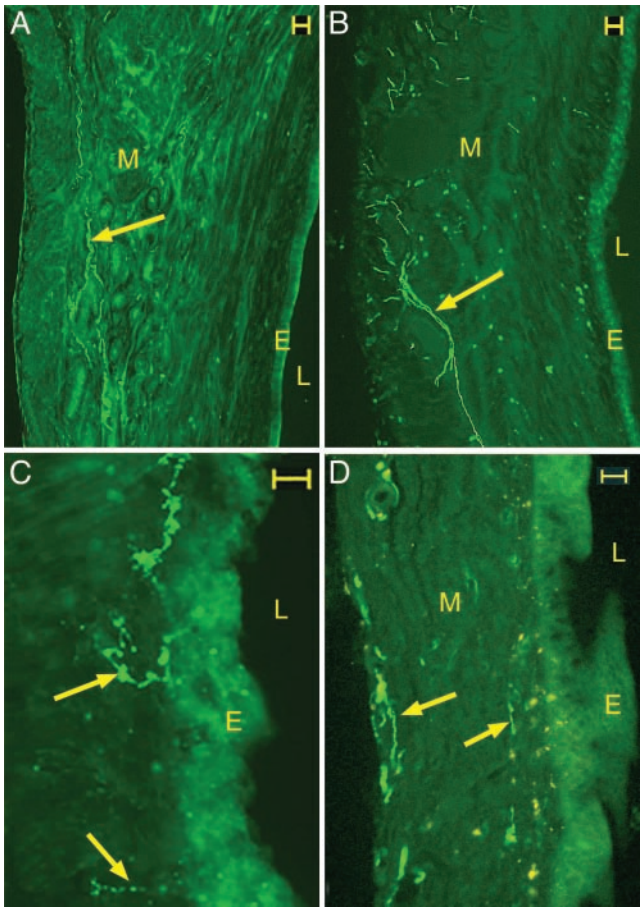

Fig. 2. Specific markers for afferent and efferent fibers in control uterus (*A*) and cyst (*B–D*) from the same rat. Sections in *A–C* were immunostained for CGRP (afferent fibers), those in *D* for VMAT (sympathetic fibers). (*A*) Uterine wall showing neurites (arrow) in the myometrium (M); E, epithelium; L, lumen. (*B*) Cyst wall showing neurites (arrow) in the myometrium (M) (compare with *A*). (*C*) Higher magnification of the cyst wall showing neurites (arrows) in the endometrium subjacent to the cyst epithelium (E) and appearing to enter the epithelium. (*D*) Cyst wall showing sympathetic neurites (arrows) in myometrium (M), near arteries and the endometrial layer. (Bar: 25 μm.)

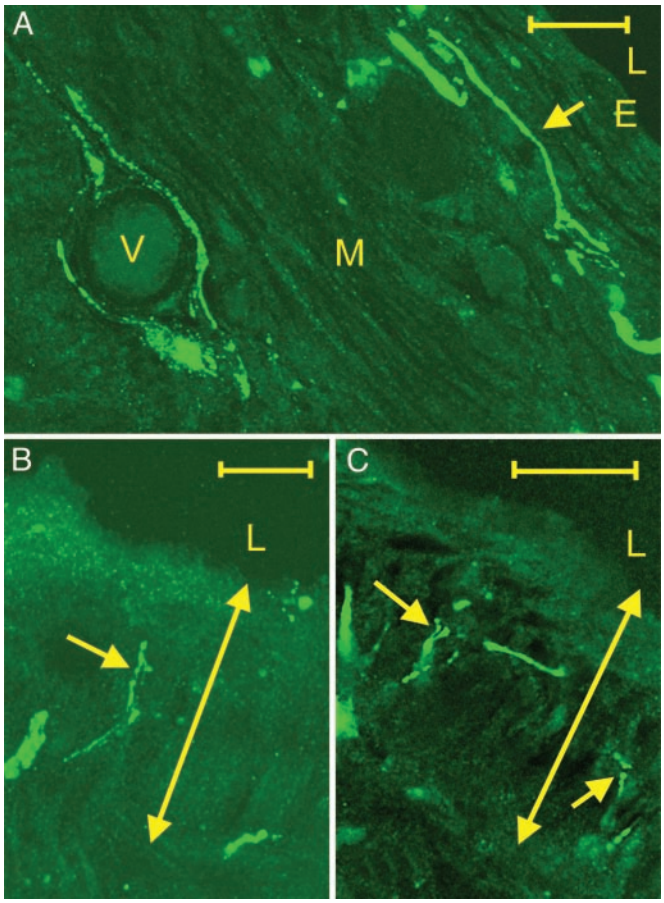

Fig. 3. Confocal microscope images of sections immunostained for specific markers for afferent fibers in a cyst. (*A* and *B*) Immunostained for SP. (*C*) Immunostained for CGRP. (*A*) Cyst wall showing neurites (arrow) in the endometrial stroma subjacent to the epithelium (E) lining the lumen (L). SP-immunoreactive neurites also closely surround the blood vessels (V) coursing through the wall and muscle layer (M) of the cyst. (*B*) Cyst wall showing individual SP-immunoreactive varicose neurites (arrow) extending into the epithelium (extent of the epithelium indicated by the double-headed arrow) lining the cyst lumen (L). (*C*) Image similar to *B* of the cyst wall showing CGRP-immunoreactive neurites (arrows) in the epithelial layer (indicated by the double-headed arrow) lining the cyst lumen (L). (Bar: 25 μm.)

encapsulated by a narrow wall of tissue consisting of an epithelium, endometrial-like stroma, smooth muscle, and adventitial-like connective tissue (Fig. 1*B*). An anchoring hilus area had developed where the vasculature and nerve supply entered/exited the cyst (Fig. 1 *B–D*).

PGP9.5. As shown by this pan neuronal marker, cysts were robustly innervated, with many neurites accompanying blood vessels as they entered the cyst at the hilus (Fig. 1 *B–D*). The neurites extended from the anchoring hilus into the nearby stromal and muscular layers (Fig. 1*D*) and eventually reached the luminal layers (Fig. 1 *D* and *E*), a pattern that mimics innervation of the eutopic uterus (13, 15, 17, 18). Moreover, neurites extended from the hilus, largely appearing to follow the course of the vasculature around the wall of the cyst.

Markers for Specific Classes of Neurites. Immunostaining for CGRP and SP was used to identify sensory neurites, whereas VMAT immunostaining was indicative of monoamine-containing sympathetic efferent neurites. As expected (13, 15, 17, 18), both types of fibers were observed in the eutopic uterus and mimicked the pattern observed using PGP9.5 in the control uterus samples (e.g., Fig. 2*A*).

Sensory and sympathetic efferent fibers also were observed in the ectopic cysts obtained from the same rats. As in eutopic uterus, their distribution pattern mimicked that observed for PGP9.5-stained neurites (Figs. 2 and 3); i.e., neurites were densest in the region where the cyst surrounded the blood vessel to which it was sutured (hilus; Fig. 1 *B* and *C*). Both types of fibers coursed with blood vessels through the cyst wall (Figs. 2*D* and 3*A*), and then small bundles and individual fibers extended into the myometrium (Fig. 2 *B* and *D*) and eventually to the endometrial stroma (Figs. 2 *C* and *D* and 3*A*). Some fibers, particularly sensory fibers expressing CGRP (Figs. 2*C* and 3*C*) and SP (Fig. 3*B*), extended small bundles and individual neurites further into the endometrial layer and even into the epithelium lining the lumen. Many of the neurites that penetrated among the epithelial cells were single varicose fibers that had the same appearance as terminal sensory receptive structures found in other hollow organs such as the bladder urothelium (19, 20).

Discussion

These findings demonstrate that the autotransplanted ectopic endometrial growths in a rat model of ENDO develop their own

robust innervation whose appearance is similar to that of the healthy uterus (13, 15, 17, 18). Furthermore, as shown by the specific markers for VMAT, CGRP, and SP, the fully grown cysts were innervated by both sympathetic efferent and sensory C and Aδ fibers. These findings are similar to those of auto- or syngeneic transplants of other organs and tissues in experimental animals. For example, sympathetic efferent reinnervation is commonly observed in transplants of the parathyroid gland (21), pancreatico-duodenum (22), lung (23), heart (24), and adrenal gland (25). Sensory innervation, although not studied as often, is also observed, for example in transplants of lung (26, 27) and heart (28). Sensory innervation is sometimes sparse, for example in transplants of the parathyroid and adrenal glands (21, 25), and may relate to the site of implantation as noted by Korsgren *et al*. (29), who found that the extent of sensory reinnervation of pancreatic islet cells was markedly different for implants in the spleen, liver, or renal subcapsule.

The observation here that the densest innervation was adjacent to blood vessels in the hilus of the fully grown cysts and that neurites appeared to follow blood vessels and extend into myometrial and epithelial layers suggests that the developing innervation occurs via sprouting of peri- and paravascular fibers that accompany the blood vessels that vascularize the growths. A similar process seems to occur with transplantation of other organs in experimental animals. For example, in studies on rats in which the parathyroid gland was autotransplanted into the renal subcapsular space, it was observed that the graft was reinnervated beginning within 1 week posttransplant mainly by sympathetic fibers located primarily around blood vessels (21). Similarly, studies in pigs have shown that the vascularization and reinnervation of skin grafts are coordinated (5). It is likely that growth factors are involved in the coordinated reinnervation and vascularization of transplants (30, 31) and may contribute to innervation of the ectopic endometrial growths. Thus, of relevance to ENDO in women, Anaf *et al*. (7) found that nerve growth factor expression was greater in deeply infiltrating adenomyotic endometrial nodules when compared with other ectopic endometrial growths and that these nodules were located closer to nerve fibers.

Regardless of the mechanisms by which the cysts become innervated, the demonstration of a robust sensory innervation by C fibers raises the distinct possibility that this innervation contributes to both the vaginal hyperalgesia and the reduced fertility that occur in this rat model (9, 10), probably via central effects, as follows. The cysts are known to contain proinflammatory cytokines, prostaglandins, and other neuroactive agents (8, 10) that could readily activate the CGRP- and SP-positive C-fiber nociceptive afferents (32, 33) found here to be located in the cyst's epithelium. Although the route by which the information conveyed by these activated fibers enters the CNS is not yet known, given the location of the cysts in the upper abdomen, it is most likely that this route will be found to include the splanchnic nerve, with fibers traveling in the superior mesenteric, inferior mesenteric, and celiac ganglia to the lower thoracic/upper lumbar segments (9, 11). Such activated input to the spinal cord could then influence the sensitivity of neurons in the lower lumbar and upper sacral segments that receive vaginal input (9, 11, 33) via the extensive communication known to exist between the two sets of segments (34), a process which has been called viscero-visceral referred hyperalgesia (35). Such actions within the caudal spinal cord could then influence activity in brain regions associated with vaginal nociception (36, 37) as well as with reproduction (38).

It is also possible that this innervation contributes to the growth and maintenance of the cysts themselves. Studies of transplanted tissues indicate that their reinnervation, particularly by sympathetic fibers, can be important not only for better functioning of transplanted tissues but also their survival (5, 31, 39, 40). Furthermore, the fact that the sensory fibers expressing SP and CGRP are anatomically intimate with the vasculature of the cyst wall and extend terminals among the epithelial cells lining the cyst lumen suggests they have both sensory and efferent functions. For example, SP and CGRP can be released from sensory fibers in an "efferent fashion" (41, 42), and both are potent vasoactive substances (43). Thus, they could play a role, along with sympathetic nerves, in regulating the vasculature of the cyst. Finally, there is evidence that both SP and CGRP have mitogenic effects on endothelial and Schwann cells (44, 45).

The rat model we used here recreates some of the signs and symptoms exhibited by women with ENDO (8–10). There are obviously, however, clear differences between this model and ENDO in women (10). For example, we do not yet know whether the rats exhibit chronic pelvic pain symptoms other than vaginal hyperalgesia. Nevertheless, our results raise the important possibility that ectopic growths in women become innervated in association with their vascularization (46). As discussed above, symptoms of ENDO, particularly pain, are notoriously wide-ranging and are not clearly related to characteristics of the endometrial growths (2, 3). It is therefore possible that if at least some growths in women become innervated, then variations in the characteristics of this innervation, such as the type and density of afferent fibers, may be the more important variable. Such afferent and efferent innervation could also contribute to maintenance of the growths, and thus, like emerging treatments aimed at reducing vascularization of ectopic endometrial growths (47, 48), represent additional new avenues for treatment.

We thank Frank Johnson and Tom Curtis for helpful advice and Megan Storey-Workley for technical assistance. This work was supported by National Institutes of Health Grants RO1 NS11892 (to K.J.B.) and RO1 NS 22526 (to R.E.P.).

1. Yoshinaga, K. & Parrott, E. C., eds. (2002) *Ann. N.Y. Acad. Sci.* **955,** 1–408.
2. Parazzini, F., Cipriani, S., Moroni, S. & Crosignani, P. G. (2001) *Hum. Reprod.* **16,** 2668–2671.
3. Chapron, C., Fauconnier, A., Dubuisson, J. B., Barakat, H., Vieira, M. & Breart, G. (2003) *Hum. Reprod.* **18,** 760–766.
4. Wu, M. Y. & Ho, H. N. (2003) *Am. J. Reprod. Immunol.* **49,** 285–296.
5. Ferretti, A., Boschi, E., Stefani, A., Spiga, S., Romanelli, M., Lemmi, M., Giovannetti, A., Longoni, B. & Mosca, F. (2003) *Life Sci.* **73,** 1985–1994.
6. Tulandi, T., Felemban, A. & Chen, M. F. (2001) *J. Am. Assoc. Gynecol. Laparosc.* **8,** 95–98.
7. Anaf, V., Simon, P., El Nakadi, I., Fayt, I., Simonart, T., Buxant, F. & Noel, J.-C. (2002) *Hum. Reprod.* **17,** 1895–1900.
8. Vernon, M. W. & Wilson, E. A. (1985) *Fertil. Steril.* **44,** 684–694.
9. Cason, A., Samuelsen, C. & Berkley, K. (2003) *J. Horm. Behav.* **44,** 123–131.
10. Sharpe-Timms, K. L. (2002) *Ann. N.Y. Acad. Sci.* **955,** 318–327.
11. Papka, R. E. & Traurig, H. H. (1993) in *Nervous Control of the Urogenital System,* ed. Maggi, C. A. (Harwood, New York), pp. 421–464.
12. Weihe, E., Schafer, M. K., Erickson, J. D. & Eiden, L. E. (1994) *J. Mol. Neurosci.* **5,** 149–164.
13. Papka, R. E., McNeill, D. L., Thompson, D. & Schmidt, H. H. W. (1995) *Cell Tissue Res.* **279,** 339–349.
14. Carlton, S. M., McNeill, D. L., Chung, K. & Coggeshall, R. E. (1987) *Neurosci. Lett.* **82,** 145–150.
15. Papka, R. E., Cotton, J. P. & Traurig, H. H. (1985) *Cell Tissue Res.* **242,** 475–490.
16. Peter, D., Liu, Y., Sternini, C., de Giorgio, R., Brecha, N. & Edwards, R. H. (1995) *J. Neurosci.* **15,** 6179–6188.
17. Papka, R. E. (1990) *Neuroscience* **39,** 459–470.
18. Zoubina, E. V., Fan, Q. & Smith, P. G. (1998) *J. Comp. Neurol.* **397,** 561–571.
19. Fowler, C. J. (2002) *Urology* **59,** 37–42.
20. Crowe, R., Vale, J., Trott, K. R., Soediono, P., Robson, T. & Burnstock, G. (1996) *J. Urol.* **156,** 2062–2066.
21. Luts, L. & Sundler, F. (1998) *Transplantation* **66,** 446–453.
22. Korsgren, O., Jansson, L., Ekblad, E. & Sundler, F. (2001) *Transplantation* **15,** 8–13.

23. Takachi, T., Maeda, M., Shirakusa, T. & Hayashida, Y. (1995) *Acta Physiol. Scand.* **154,** 43–50.
24. Murphy, D. A., Thompson, G. W., Ardell, J. L., McCraty, R., Stevenson, R. S., Sangalang, V. E., Cardinal, R., Wilkinson, M., Craig, S., Smith, F. M., *et al.* (2000) *Ann. Thorac. Surg.* **69,** 1769–1781.
25. Ulrich-Lai, Y. M. & Engeland, W. C. (2000) *J. Neuroendocrinol.* **12,** 881–893.
26. Kawaguchi, A. T., Shirai, M., Yamano, M., Ishibashi-Ueda, H., Yamatodani, A. & Kawashima, Y. (1998) *J. Heart Lung Transplant.* **17,** 341–348.
27. Buvry, A., Yang, Y. R., Tavakoli, R. & Frossard, N. (1999) *Am. J. Respir. Cell Mol. Biol.* **20,** 1268–1273.
28. Mohanty, P. K., Thames, M. D., Capehart, J. R., Kawaguchi, A., Ballon, B. & Lower, R. R. (1986) *J. Am. Coll. Cardiol.* **7,** 414–418.
29. Korsgren, O., Jansson, L., Andersson, A. & Sundler, F. (1993) *Transplantation* **56,** 138–143.
30. Roush, W. (1998) *Science* **279,** 2042.
31. Reimer, M. K., Mokshagundam, S. P., Wyler, K., Sundler, F., Ahren, B. & Stagner, J. I. (2003) *Pancreas* **26,** 392–397.
32. Coleridge, H. M., Coleridge, J. C., Ginzel, K. H., Baker, D. G., Banzett, R. B. & Morrison, M. A. (1976) *Nature* **264,** 451–453.
33. Berkley, K. J., Robbins, A. & Sato, Y. (1993) *J. Neurophysiol.* **69,** 533–544.
34. Wall, P. D., Hubscher, C. H. & Berkley, K. J. (1993) *Brain Res.* **622,** 71–78.
35. Giamberardino, M. A., Berkley, K. J., Affaitati, G., Lerza, R., Centurione, L., Lapenna, D. & Vecchiet, L. (2002) *Pain* **95,** 247–257.
36. Berkley, K. J., Hubscher, C. H. & Wall, P. D. (1993) *J. Neurophysiol.* **69,** 545–556.
37. Berkley, K. J., Guilbaud, G., Benoist, J.-M. & Gautron, M. (1993) *J. Neurophysiol.* **69,** 557–568.
38. Kawakami, M. & Ohno, M. N. (1983) *Acta Morphol. Hung.* **31,** 117–136.
39. Wu, W., Scott, D. E. & Reiter, R. J. (1993) *Exp. Neurol.* **122,** 88–99.
40. Bengel, F. M., Ueberfuhr, P. & Schwaiger, M. (2001) *N. Engl. J. Med.* **345,** 1914–1915.
41. Maggi, C. A. & Meli, A. (1988) *Gen. Pharmacol.* **19,** 1–43.
42. Holzer, P. (1988) *Neuroscience* **24,** 739–768.
43. Brain, S. D. (1997) *Immunopharmacology* **37,** 133–152.
44. Cheng, L., Khan, M. & Mudge, A. W. (1995) *J. Cell Biol.* **129,** 789–796.
45. Haegerstrand, A., Dalsgaard, C. J., Jonzon, B., Larsson, O. & Nilsson, J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3299–3303.
46. Taylor, R. N., Lebovic, D. I. & Mueller, M. D. (2002) *Ann. N.Y. Acad. Sci.* **955,** 89–100.
47. Hull, M. L., Charnock-Jones, D. S., Chan, C. L., Bruner-Tran, K. L., Osteen, K. G., Tom, B. D., Fan, T. P. & Smith, S. K. (2003) *J. Clin. Endocrinol. Metab.* **88,** 2889–2899.
48. Nap, A. W., Griffioen, A. W., Dunselman, G. A., Bouma-Ter Steege, J. C., Thijssen, V. L., Evers, J. L. & Groothuis, P. G. (2004) *J. Clin. Endocrinol. Metab.* **89,** 1089–1095.